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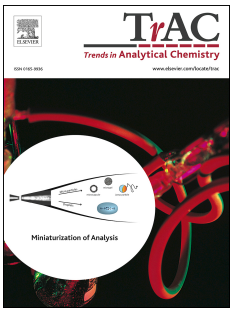

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Developing aptasensors for forensic analysis

James Gooch [a], Barbara Daniel [a], Mark Parkin [a], Nunzianda Frascione [a,*]

[a] *Analytical and Environmental Sciences Division, King's College London, 150 Stamford Street, London, SE1 9NH, UK*

** Corresponding author. Tel.: +44 20 7848 4978*
E-mail address: nunzianda.frascione@kcl.ac.uk (N. Frascione).

Abstract

Aptamer-based biosensors may be of significant benefit to forensic analysis by allowing the rapid, sensitive and specific detection of molecular targets relevant to criminal investigation. However, despite the production efficiency, stability and cost effectiveness of aptamer recognition moieties, aptasensors have yet to find commercial employment within any area of forensic science. This review therefore attempts to encourage aptasensor development by initially identifying the methods of selection, sequence analysis and affinity measurement most appropriate for the discovery of suitable aptamers against analytes of forensic interest. A range of optical, electrochemical and mass-sensitive transduction platforms that may be considered amenable to current forensic testing procedures are then discussed. The specific analytical disciplines in which aptasensing technology is likely to be of greatest value, including forensic drug analysis, forensic toxicology and biological evidence and explosives detection are lastly highlighted to stimulate researchers to consider the development of sensors towards these particular target types.

Keywords

Aptasensors; Aptamers; Biosensors; Forensic; Analytical Science; DNA

1. Introduction

Forensic science may be considered one of the broadest analytical disciplines due to the extensive spectrum of analytes and sample types used to add value to a criminal investigation. For example, in the field of human identification alone, examinable material may range from simplistic visual patterns (e.g. fingermarks) to complex biological molecules (e.g. DNA) [1]. Challenges associated with the growing variety of analytical techniques and instrumentation required by forensic laboratories to meet comprehensive testing demands have prompted the search for new and flexible methods that are able to detect, identify and quantify analytes of forensic interest.

Immunological analysis methods have long been an integral part of forensic serological and toxicological screening processes [2]. However, with the recent discovery of aptamer-based recognition, research groups are beginning to question the efficiency of the ELISA and lateral flow strip testing strategy's currently employed by forensic laboratories. Nucleic acid aptamers are short single-stranded DNA or RNA sequences that are able to undergo selective antigen association as a result of three-dimensional structure formation [3]. These structures (usually a combination of k-turn, loop, pseudoknot and quadruplex motifs) facilitate intermolecular interaction with targets via van der Waal forces, hydrogen bonding and aromatic ring stacking [4]. Through the *in vitro* enrichment of random oligonucleotide libraries (consisting of approximately 10^{12}-10^{15} individual sequences) aptamers may be developed towards almost any small molecule [5], virus [6], large protein [7] or whole-cell target [8], giving potential for their use as recognition moieties in the analysis of diverse forensic samples.

While analogous to antibodies in terms of binding affinity (often displaying K_d values in the nanomolar or picomolar range [9]), aptamers possess a number of key advantages over their protein counterparts. Once selected, aptamer sequences can be mass-produced using automated solid-phase synthesis techniques, resulting in the production of highly purified oligonucleotides within a number of hours and at a fraction of the cost of biological antibody generation methods [10]. Aptamers also display greater thermal stability affording long shelf-lives without loss of activity, easy transport and storage and ability to return to a native confirmation being subjected to high-temperature assay conditions [11].

Although demonstrating success as effective therapeutic agents [12], aptamers, since their discovery, have been predominately developed as recognition molecules for use in a range of biological chemical analysis techniques. This has mainly focused on the use of immobilized aptamers within traditional ELISA, western blot, flow cytometry and lateral flow assays as replacements for expensive and cumbersome antibody moieties [10]. In addition, aptamers have also found beneficial application with the field of separation science, having been used to successfully resolve enantiomeric molecules within High Performance Liquid Chromatography (HPLC) systems by the inclusion stereospecific aptamers on solid chromatographic supports [13].

Nevertheless, Hamaguchi *et al.* argue that the most powerful application of nucleic acid aptamers is within analytical biosensing platforms [14]. Biosensors are compact devices capable of the real-time transduction of biological interaction events into a number of measurable signal outputs [15]. Some authors have recently recognized the potential of biosensors to provide the highly specific detection and quantification of forensically relevant materials (such as body fluids, drugs, explosives and toxins) without the need for extensive sample processing steps [16-18]. Displaying significant

conformational changes upon target binding and allowing an extensive range of chemical modifications (including the incorporation of various optical, electrochemical or nanoparticle reporters [19]) at various sites without loss of binding affinity, aptamers may be considered as ideal recognition moieties for use within molecular sensing purposes [20].

Table 1 - SELEX protocols that may be used in the selection of aptamers against forensic targets

Protocol	Mechanism	Advantages	Reference
Capillary-Electrophoresis SELEX	Aptamer-target complexes are separated from unbound nucleotide sequences according to electrophoretic mobility.	- Selection may be completed within 2-4 rounds. - Useful for small molecule targets.	[21]
Capture-SELEX	Library sequences are displaced from magnetic beads by target binding. Unbound ligands are then removed magnetically.	- Aptamers display large structural changes. - Useful for small molecule targets.	[22]
Cell-SELEX	A panel of membrane biomarker-specific aptamer sequences is produced as a result of exposure to live cell targets.	- Counter-selection used to increase specificity. - Selection performed on targets in native state.	[23]
FluMag SELEX	Complexes formed between bead-immobilized analytes and fluorescent library sequences are collected magnetically.	- Selection rounds and dissociation values may be quantified by fluorescence measurement.	[24]
Graphene Oxide (GO) SELEX	Graphene oxide is used to adsorb and separate unbound ssDNA sequences from aptamer-target complexes in solution.	- Requires less than 5 rounds of selection. - Useful for small molecule targets.	[25]
In-Silico SELEX	Theoretical oligonucleotide libraries are screened against a target using computational tools to identify potential binders.	- Atomic-level mechanisms of nucleotide-target binding can easily be determined.	[26]
Microfluidic (M) SELEX	Ligands bound to magnetic or sol-gel bead-conjugated targets are purified by continuous washing within a microchannel.	- Lower target molecules and reagent volumes are required as a result of miniaturization.	[27]
MonoLEX	Affinity columns used to partition aptamer-target complexes are physically segregated to elute highest affinity sequences.	- Requires a single round of selection. - Diminishes competition between sequences.	[28]
NanoSelection	High affinity aptamers are detected and recovered via the use of fluorescence and Atomic Force Microscopy (AFM).	- Requires a single round of selection. - Binding affinity may be measured by AFM.	[29]

Despite great promise, aptasensors (or indeed any form of aptamer technology) have yet to find commercial employment within the field of forensics. This lack of success has been attributed to a number of factors, including the limited number of aptamer sequences raised against analytically relevant targets [20], the need for further investigation into the use of developed sensors within complex matrices [17] and the high investment already made by analytical laboratories in antibody-based testing methods [30]. This review therefore attempts to outline the current state of aptasensor development processes, with a specific focus on those techniques most likely to aid the construction of forensic analyte sensing devices. Recent advances in aptamer selection, sequencing and affinity testing are first explored, along with signal transduction mechanisms most amenable to current forensic testing capabilities. A number of forensic disciplines liable to benefit from aptasensor application are lastly identified in the hope of encouraging experts within the field of biosensor design to produce aptasensors towards valuable evidential targets.

2. Aptamer development

2.1 Aptamer selection

In recent years, a substantial number of modifications have been made to the SELEX (Systematic Evolution of Ligands by EXponential enrichment) protocol initially developed by Gold's [31] and Szostak's [32] groups. While generally based on the same library incubation, target binding and sequence amplification principles exploited within the original method, these modified techniques differ in their approach to the separation and removal of non-specific ligands [33]. By altering the means in which aptamer–target complexes are partitioned from unbound oligonucleotides, aptamers may be selected against particular target types with a higher specificity and affinity than would be possible using conventional SELEX protocols [34]. As the physical properties of forensically relevant targets are extremely diverse, it is vital that an optimal selection method is chosen and carried out prior to aptasensor development. A summary of SELEX protocols that may be useful in the selection of aptamers against forensically relevant targets can be found in Table 1.

These modifications are especially pertinent in the selection of aptamers towards low-molecular weight forensic analytes (e.g. toxic chemicals and explosives), where separation is problematic due to the similar masses of bound complexes and unbound nucleic acid sequences [35]. The immobilization of such targets to a solid support surface (e.g. sepharose, agarose or magnetic beads) may be used to increase separation efficiency but can also result in the amplification of non-specific sequences that bind to the support matrix or immobilization linkers [5]. Furthermore, the creation of a chemical link between the small molecule target and the solid support can introduce an unfavorable selection

bias through the removal of least one potential aptamer binding site. Fortunately, a number of SELEX processes that allow for the selection of aptamers against small-molecules without the need for target immobilization have recently been developed.

Capture-SELEX (also known as structure-switching SELEX), involves the conjugation of oligonucleotide libraries themselves to DNA-functionalized magnetic particles by use of a complimentary 'docking' sequence [22, 36]. When incubated with a target molecule, sequences that undergo conformational changes as a result of binding are displaced from the beads (which can then be removed with unbound ligands through the application of a magnetic field). Aptamers isolated using this capture method may be considered particularly attractive recognition moieties for use in analytical biosensing platforms due to their significant structure-switching abilities [37].

Another immobilization-free method, GO-SELEX, makes use of the ability of graphene oxide (GO) to bind single stranded DNA through π-π stacking interactions [25]. In this technique, target molecules are first incubated with an oligonucleotide library in solution, allowing binding to occur. Unbound sequences are then adsorbed onto the surface of the graphene oxide, allowing separation from aptamer-target complexes via centrifugation. Ethanol precipitation is finally used to purify and recover bound ssDNA sequences. As partitioning is purely based on interaction between DNA libraries and graphene, GO-SELEX is largely independent of target size (and has already been successfully employed to produce aptamer sequences towards low-molecular weight pesticide compounds [38]). One class of forensic analytes that may also benefit from aptamer- sensing application is that of whole-cells. The immunological analysis of cellular material is currently undertaken by forensic laboratories as a method of confirming the identity of biological fluid deposits left behind at crime scenes [2]. Unlike conventional SELEX procedures, the selection of aptamers against whole-cells does not require the use of a single highly purified (or even known) target. First described by Morris *et al.*, Cell-SELEX instead involves concurrent binding of library oligonucleotides to multiple biomarkers exposed on the surface of intact live cells [23]. As a result, this approach generates a panel of aptamer sequences (each targeting a particular membrane protein or lipid) that can then be used for cell recognition purposes [39]. The use of live targets within Cell-SELEX ensures that aptamers are raised towards the native conformational structure of such membrane molecules, allowing for the high-affinity binding of natural cellular material during subsequent analysis [8]. In comparison to other selection protocols, the partitioning of unbound nucleotides within Cell-SELEX is relatively simplistic and may be achieved by basic centrifugation or washing steps [40]. A counter-selection stage, in which enriched libraries are incubated with a negative control cell line, is also often included in cell-selection protocols to remove non-specific ligands that bind to universally shared membrane motifs [41]. Cell-SELEX techniques have in fact already been used to produce aptamers against sperm [42] and red-blood cell targets [23], which may already be of potential use within forensic assays for the analysis of biological fluids.

2.2 Sequence analysis

Following sufficient enrichment, aptamer pools are sequenced in order to elucidate the nucleotide structures of high-affinity binders [33]. Conventionally, this process was performed via the ligation of selected sequences into commercially available cloning vectors, which are then transformed into competent bacterial cell colonies. Approximately 50-100 individual clones (each possessing one aptamer sequence) are then recovered before plasmids are extracted and subjected to standard Sanger sequencing [43]. However, whilst able to indicate the most abundant aptamers present within an enriched pool, these cloning techniques only involve the sequencing of a small fraction of the total binders obtained during selection (which may be up to 1,000,000 sequences) [44]. As a result, the majority of new aptamer development protocols now make use of next generation sequencing (NGS) platforms for the comprehensive analysis of potential binding candidates [45]. Such high-throughput methods not only allow the discovery of millions of sequences from enriched pools without laborious cloning processes, but can also be used to shorten selection processes by identifying aptamers during early SELEX rounds [46].

Fig. 1 – Workflow of the Illumina sequencing-by-synthesis approach – Specialist adapter sequences are first ligated to the end of aptamers obtained from SELEX-enriched pools. These adapters aid the anchoring of selected oligonucleotides to the surface of a flow cell, allowing solid-phase bridge amplification. Fluorescently labeled nucleotides are then added to the cell, which is imaged after the incorporation of each base into DNA strands. The emission wavelengths of each nucleotide dye are then monitored in order to determine aptamer sequences.

Commercially available NGS platforms previously used for aptamer discovery include Ion Torrent's Personal Genome Machine (PGM) [47] Illumina's Genome Analyzer/HiSeq system [48, 49], Roche's GS FLX system [50, 51] and Applied Biosystem's SOLiD system [52]. However, with extensive sequence reads and higher total read lengths, a greater level of detail on the structural features of target binders may be provided by Illumina instruments, making them the most preferable choice for aptamer sequencing (Fig. 1) [43]. Furthermore, these platforms, based upon the principle of cyclic

reversible termination, are already widely used within the forensic community for the analysis of human genomic material [53] and may therefore be readily applied by forensic researchers to determine aptamer sequences for the recognition of forensic analytes.

While NGS use may significantly enhance the resolution and sampling depth of enriched aptamer pools, additional computational bioinformatic tools are currently required to process the sheer quantity of raw sequence data obtained [45]. Web-based and offline preprocessing software, such as Galaxy [54], cutadapt [55] and AptaTools [56] are often used to first isolate variable aptamer regions of a defined length by removing adapter and constant region sequences. Further programs are then employed to filter this data in order to narrow down aptamer candidates for subsequent experimental testing. The FASTaptamer toolkit achieves such filtering by monitoring the relative enrichment of specific aptamer sequences between selection rounds [57], whereas APTANI examines both the total read counts of each sequence as well as shared structural homology between sequences [58]. After aptamer discovery has taken place, sequences may be further analysed by programs such as *mfold* [59] to provide more information on secondary oligonucleotide structure.

2.3 Affinity measurement

In order to ensure adequate aptasensor performance, it is vital that integrated aptamers demonstrate strong affinity towards target molecules [60]. Aptamer affinity is usually expressed in terms of a dissociation constant (K_d), the value of which may be determined by a range of separation-based, mass-sensitive, spectroscopic or other label-free techniques [5]. A summary of methods that may be able to assess the performance of aptamers with potential use in forensic testing may be found in Table 2.

Table 2 – Techniques that may be used to determine the affinity of aptamers against forensic targets

Technique	Type	Mechanism	Reference
Affinity Chromatography	Separation	- Binding is measured after labeled aptamers or target molecules are incubated with corresponding components that are conjugated to a solid support (typically agarose or magnetic beads).	[61]
Capillary Electrophoresis	Separation	- Concentrations of aptamers, targets and aptamer-target complexes are determined after size and charge-based separation. Fluorescent labeling of each component is often required for detection.	[21]
Equilibrium Dialysis	Separation	- Unbound ligands within an aptamer-target mixture are allowed to diffuse through a semi-permeable membrane before being quantified.	[62]
Gel Electrophoresis	Separation	- Like capillary electrophoresis, binding components are separated by size and charge but by a non-denaturing agarose or polyacrylamide gel. Visualization is required to observe isolated components.	[63]
Ultrafiltration	Separation	- Unbound ligands are measured after partitioning across a membrane (typically nitrocellulose) under applied pressure, vacuum or centrifugation.	[64]
Back-Scattering Interferometry (BSI)	Spectroscopic	- Changes in the refractive index of a sample through the association of aptamer-target complexes are detected within a microfluidic channel.	[65]
Circular Dichroism (CD)	Spectroscopic	- Differences in the absorption of left and right circularly polarized light are monitored during the titration of DNA against increasing concentrations of target.	[66]
Fluorescence Intensity	Spectroscopic	- The increase/decrease in fluorescence intensity of targets or labeled aptamers as a result of complex formation is measured via spectrofluorometry.	[67]
Fluorescence Polarization	Spectroscopic	- The rotational diffusion of fluorescent dyes conjugated to aptamers or targets is decreased as a result of binding. Subsequent increases in signal polarization are then monitored.	[68]
UV-Vis Absorption	Spectroscopic	- Affinity is inferred from variations in the wavelength or intensity of UV-Vis absorption of aptamers or targets upon binding.	[7]
Quartz Crystal Microbalance	Mass-Based	- Accumulation of aptamer-target complexes on the surface of functionalized piezoelectric crystals results in a measurable decrease in resonance frequency.	[69]
Surface Plasmon Resonance	Mass-Based	- Ligands immobilized on a sensor flow cell are subjected to an aqueous flow of binding partners. The formation of target complexes then results in a change in refractive index near the sensor surface.	[70]
DNase Footprinting	Other	- The relative levels of aptamers protected from digestion (by association with varying concentrations of target molecules) are determined after treatment with DNase I enzymes.	[71]
Isothermal Titration Calorimtery (ITC)	Other	- The amount of energy required to maintain the temperature of a cell is monitored during exothermic aptamer-complex binding.	[72]

Variations in the size and physical properties of forensically relevant analytes make the evaluation of aptamer-target affinities challenging. Much like similar SELEX processes, measuring K_d via the separation of unbound ligands from aptamer-target complexes is often problematic for compounds of a smaller molecular weight than nucleotide binding sequences. Other techniques, such as fluorescence polarization or surface plasmon resonance (SPR), are additionally disadvantaged by the chemical labeling or immobilization of aptamers or targets, which may reduce K_d calculation accuracy by altering binding interactions [5].

A resolution to these issues may be provided by isothermal titration calorimetry (ITC), a label-free solution-based method that allows the characterization of binding energy by monitoring temperature increases during complex formation [72]. ITC methods are currently considered the 'gold standard' for quantifying biomolecular interactions and have already been used to assess the affinity of aptamers towards several forensically relevant targets, including cocaine [73] and organophosphate pesticides [74]. In this technique, targets are titrated into a cell containing an aptamer of interest and allowed to react. Heat released as a result of exothermic binding processes is monitored and compared to an identical reference cell containing buffer or water. Power required by the calorimeter to maintain equal temperature between the cells at each molar ratio of target/aptamer may then be used to construct a binding isotherm, allowing affinity to be determined [75]. However, while ITC may provide detailed information on the thermodynamic parameters of aptamer-target interactions (such as changes in entropy, enthalpy and Gibb's energy), relatively high amounts of target are required for detectable amounts of heat to be generated [7]. This may be considered problematic for some forensic analytes that are expensive to purchase or only available from commercial providers in low concentrations (although micro and nano-ITC instruments may be utilised to overcome these challenges).

3. Biosensing platforms

3.1 Optical

Reported by Kleinjung *et al.* in 1998, the first biosensor to use aptamers as moieties for target recognition involved the competitive binding of L-adenosine and FITC-labeled analogues to an RNA ligand immobilized on the surface of an optical fibre [76]. As a result, the vast majority of early aptasensing platforms also focused on the use of optical methods to signal biological interaction occurrence [77]. Such sensing generally relies on the structural transitions that aptamers undergo during target binding to create measurable variations in the spectroscopic properties of optical transduction components [19]. These components (typically organic dyes, luminophores, nanoparticles or conjugated polymers) may be incorporated into conformationally labile regions of oligonucleotide sequences by covalent attachment or as 'label-free' reporters by indirect intercalation [78]. Subsequent alterations in the microenvironment of these reporters as a result of aptamer folding may then prompt changes in the intensity, wavelength or anisotropy of label emissions [20]. Alternatively, multiple reporters can be used to achieve transduction through distance-dependent fluorescence (FRET) or chemiluminescence (CRET) resonance energy transfer processes [79].

Fig. 2 – Schema of aptamer-based sensing formats – a) Optical transduction. Aptamers labeled with fluorescence reporters (F) are hybridized to quencher (Q)-conjugated DNA sequences, which absorb emission via FRET. Upon binding to targets (T), aptamers separate from complimentary strands, allowing fluorescence to be restored. b) Electrochemical transduction. Changes in aptamer confirmation as a result of target interaction allow redox-active labels to interact with the surface of electrodes to produce an electrical signal. c) Mass-sensitive. An increase in the mass of surface-immobilized binders through aptamer-target complex formation is detected either optically or electrically.

Two popular forms of optical aptasensing include aptamer-beacons and hybridized DNA displacement assays [80]. Based on the conventional molecular-beacon format for the detection of specific DNA molecules, aptamer-beacons are constructed by the addition of short complementary nucleotides (alternately labeled with a fluorophore or quencher) to each end of a specific aptamer sequence [14]. Under normal conditions these nucleotides allow the aptamer to take on a closed hairpin structure, bringing the quencher and fluorophore moieties within close proximity and restricting fluorescence output. However, in the presence of target molecules, aptamer interaction causes the hairpin to unfold, resulting in the production of a 'signal-on' fluorescence emission [80]. Hybridized DNA displacement assays (Fig. 2a) work via a similar premise but instead employ a separate quencher-labeled antisense sequence bound to fluorescent aptamers through Watson-Crick base pairing that then dissociates in the presence of higher affinity target analytes [81].

One of the main advantages of optical sensing is the ability to offer real-time analyte detection without extensive sample processing steps or specialized equipment [82]. Such advantages may give the potential for optical aptasensors to be used as chemical reagents for the simultaneous detection and identification of latent (i.e. non-visible) evidence

deposited at crime scenes or on evidential items. This concept has already been explored within Li *et al.* in which two aptamers labeled with emitting gold nanoparticles (Au-NPs) reassembled in the presence of cocaine doped within latent fingermarks [83]. While the adherence of the nanoparticles themselves to deposited marks was sufficient to visualize identifiable ridge detail, aggregation of the Au-NPs as a result of cocaine binding also resulted in a shift in emission wavelength of scattered light from 550-580nm.

3.2 Electrochemical

Cho *et al.* note that despite the significant amount of optical detection platforms reported during the early stages of aptasensor research, greater attention is now being paid to the use of electrochemical strategies for the transduction of aptamer interactions [20]. Biosensing assays incorporating such methods are becoming increasingly attractive to researchers due to their relative portability, ease of operation, robustness and low cost (to both develop and operate) [84]. Electrochemical aptasensors are further benefitted by excellent sensitivities and can be used in conjunction with a number of electrical signal amplification techniques (such a biocatalytic labeling) to provide extremely low limits of analyte detection [85]. For example, Hanson *et al*. employed the use of a single-step replacement aptasensor exploiting electrochemical nanoparticle stripping-based signal amplification for the measurement of thrombin at ultrasensitive attomole levels [86].

While electrochemical aptasensors may make use of amperometric, potentiometric, conductometric, impedimetric and semiconductor field-effect principles, signals are generally derived from changes in electric current as a result of aptamer-mediated redox reactions occurring at the surface of an electrode [84]. Much like optical aptasensing mechanisms, these reactions are generated by reporter molecules incorporated within aptamer sequences, which are then bought closer to or further away from electrodes as a result of target binding interactions [79]. Popular reporters for electrochemical aptasensors include methylene blue (MB), ferrocene, ferricyanide, ruthenium complexes, enzymes, quantum dots (QDs) and metal nanoparticles [87].

According to Han *et al.*, electrochemical aptasensor assays may be designed in four broad formats: target-induced structure switching (TISS), sandwich, target-induced displacement (TID) or competitive replacement [88]. TISS assays exploit the ability of surface-immobilized aptamers to form rigid tertiary structures in the presence of analytes in order to change the proximity of signaling moieties in relation to an electrode (Fig. 2b). Sandwich assays conversely involve the assembly of a complex between an immobilized primary aptamer, a target, and a second reporter-labeled recognition molecule (which may be an aptamer or an antibody). In TID sensors, complementary nucleotide sequences are instead immobilized and are used to anchor labeled aptamers to electrodes via base pairing. Upon target interaction these aptamers dissociate, causing a decrease in electron transfer (eT) signals. Competitive replacement lastly involves the incubation of targets with reporter-attached analytes, which then compete for aptamer binding space. As a result, signal intensities obtained from competitive replacement assays are inversely proportional to the amount of target present within a sample.

Electrochemical detection platforms may be considered especially amenable to forensic analysis due to their excellent performance in turbid matrices [89]. Such sensing platforms are able to negate the effects of optically absorbing and fluorescent molecules present within complex samples (that often interfere with spectroscopic analysis) and therefore represent great potential as toxicological assays for the detection of trace compounds within biological matrices. Work conducted by Baker *et al.* has already proven this capability through the production of an electrochemical TISS aptasensor that was successfully able to detect micromolar concentrations of cocaine in saliva and blood serum samples [90].

3.3 Mass-sensitive

Unlike both optical and electrochemical transduction mechanisms, mass-sensitive aptasensing techniques do not utilise the conformational changes that aptamers undertake in order to indicate biological interaction events [20]. Instead, sequences are tethered to a variety of solid supports and used as simple capture ligands to create discernible increases in mass at sensor surfaces upon target binding (Fig. 2c). As a result, such methods do not require the use of molecular reporters to generate detectable signals and are therefore classified as ‘label-free’ techniques [79]. Popular mass-based aptasensing formats include: SPR, surface acoustic wave (SAW), quartz crystal microbalance (QCM) and microcantilever assays [79].

Employing the same principles for the characterization of binding affinity constants, SPR methods have also found use as platforms for the aptamer-based detection of numerous target compounds [91]. One of the most frequently used instruments for SPR sensing is the Biacore™ system manufactured by GE Healthcare [92]. In this system, aptamers are first covalently immobilized onto a thin gold film attached to a glass slide, which is then illuminated by monochromatic p-polarised light. Solutions containing an analyte of interest are then introduced into the sensor at a continuous flow through microfluidic channels. As these analytes interact with aptamer sequences, increases in mass bound to the film surface cause changes in the refractive index of incident light, which is then registered by the instrument [93].

SAW and QCM-based aptasensors both involve the generation and detection of acoustic waves by electrodes patterned on the surface of aptamer-functionalized piezoelectric crystals [94]. The propagation speed of such waves (either on the surface or in the bulk of crystals for QCM and SAW respectively) is highly influenced by mass associated with the crystal itself. Increases in this mass as a result of aptamer-target binding subsequently cause a reduction in crystal resonance frequencies, which are then observed by electrical means [20]. Whilst these devices are normally only applicable for the sensing of large analytes such as proteins or cells (which provide more measurable mass changes) a number of modified protocols, such as QCM with dissipation monitoring (QCM-D), have been developed to allow the aptamer-based detection of lower molecular weight analytes [35].

Microcantilever assays typically consist of thin silicon or polymer-based micromechanical beams (approximately one micron thick), which respond to changes in physical stress [95]. A gold coating is often applied to one side of the beam in order to allow the immobilization of biological receptors, including aptamers, onto cantilever surfaces [96]. The binding of target molecules to these receptors creates stress differences between the functionalized and non-functionalized sides of the surface, causing the cantilever to bend by a matter of nanometres [97]. The degree of bending (also known as cantilever deflection or $\Delta\chi$) is then detected optically and compared to a reference cantilever containing non-interacting nucleotide sequences [98]. As this deflection is directly proportional to the amount of target present within a sample, microcantilever methods represent a great opportunity for the quantitative sensing of forensically relevant analytes.

4. Aptasensor applications

4.1 Biological evidence

Chemical reagents used for the detection and identification of biological evidence (i.e. body fluids and fingermarks) are currently limited by issues of low specificity and sensitivity, environmental and safety concerns, ease of application and effect on downstream DNA profiling processes [16, 99]. Other antibody-based devices (such as immunochromatographic test cartridges) are conversely able to provide absolute confirmation of body fluid identity with a sufficient degree of sensitivity and safety, but cannot be used to locate latent stains on evidential surfaces [100]. As previously mentioned, optical aptasensing strategies may have the opportunity to overcome these challenges by allowing the simultaneous detection and highly specific identification of biological evidence *in situ*.

Fig. 3 – Schema of a FRET-based aptasensor for the detection of thrombin developed by Wang *et al.* [101]. Here, 5' amino-modified aptamer sequences are first covalently linked to poly-acrylic acid (PAA)-functionalized up-conversion phosphors (UCP's). Subsequent incubation with carbon nanoparticles results in quenching of the sensor through aptamer-abridged fluorescence resonance energy transfer (FRET). This sensor demonstrated a linear detection range of 0.5-20 nM and was even used to detect Thrombin concentrations of 0.25 nM within spiked human serum samples.

Furthermore, a number of optical aptasensors have already been constructed towards biomarkers that may be (or currently are) used for the confirmation of body fluid presence. For example, Kong *et al.* recently reported the production of a fluorescent aptasensor towards prostate specific antigen (PSA), a serine protease enzyme already used in forensic analysis for the immunological detection of seminal fluid [102]. In this assay, fluorophore-labeled aptamers adsorbed onto the surface of emission-quenching MoS_2 nanosheets were used to detect sub-nanogram concentrations of PSA after dissociation upon target binding.

Since its isolation by Bock *et al.* in 1992, the thrombin-binding aptamer has become one of the most commonly exploited DNA receptor sequences for the construction of new aptamer-based sensing platforms [103]. As a result, a number of these aptasensing assays may find application in the specific detection of thrombin for the purposes of identifying bloodstains deposited on evidential surfaces. One such sensor, developed by Wang *et al*., involves the use of aptamer-functionalized upconverting phosphors, which are initially quenched by the binding of carbon nanoparticles to DNA strands through π-π stacking interactions (Fig. 3). In the presence of thrombin, these nanoparticles dissociate from ligand sequences, relieving FRET quenching effects and producing 'signal-on' fluorescence emission. [101].

4.2 Forensic drug analysis

Routinely employed methods for detecting trace quantities of illicit drugs include presumptive chemical tests, immunoassays and a wide selection of chromatographic techniques [104]. With a high sensitivity, selectivity and reliability, gas chromatography coupled with mass spectrometry (GC-MS) or liquid chromatography coupled with mass spectrometry (LC-MS) is currently considered the 'gold-standard' for the forensic analysis of bulk drug samples.

However, such approaches are costly and may be considered inappropriate for high-throughput analysis due to extensive run times. Rapid, cheap and portable drug-screening assays based on aptasensor technology are therefore likely to be welcomed by the forensic community.

Fig. 4 – Schema of a colorimetric Au-NP aptasensor for the detection of methamphetamine developed by Yarbakht and Nikkhah [105]. In this assay, aptamers are initially adsorbed onto the surface of gold nanoparticles (au-NP's) through hydrophobic and electrostatic interactions to stabilize them within solution. However, in the presence of methamphetamine (MA) or 3,4-Methylenedioxymethamphetamine (MDMA), alterations in the structural conformation of the aptamers allow salt-induced aggregation of the particles to occur, prompting surface plasmon resonance-based colour changes. A purely visual detection of MA was found to occur at concentrations as low as 5 mM.

Much like the thrombin-binding sequence previously discussed, a cocaine-specific aptamer constructed by Stojanovic *et al.* [106] has also been extensively used as a model ligand for proof-of-concept aptasensing assays. Demonstrated by Hilton *et al*., a particularly sensitive example of such assays involved the target-induced displacement of Dabcyl quencher-attached complementary oligonucleotides from carboxyfluorescein (FAM) labeled aptamers immobilized within microfluidic channels. This assay was subsequently shown to enable the detection of cocaine at picomolar levels, without the need for sample cleanup and derivatization processes required for GC-MS analysis [107].
Beyond cocaine-based assays, initial explorations are also being made into the use of aptasensing platforms for the detection of amphetamine derivatives. Yarbakht and Nikkhah recently exploited the ability of single stranded aptamers to shield Au-NPs from salt-induced aggregation in order to allow the colorimetric signaling of MA and MDMA presence [105]. In this sensor (Fig. 4), an aptamer able to bind both MA and MDMA is incubated with a given sample. If target molecules are contained within the sample, conformational changes in the aptamer take place as a result of binding. Such changes prevent the association of aptamer sequences with Au-NPs, which then aggregate upon salt addition, turning the colour of sample solutions from red to blue. If target molecules are absent from the sample, aptamers are instead able to interact freely with AuNPs, protecting them from aggregation.

In an alternative transduction approach, Huang *et al.* [108] were able to successfully develop an electrochemical biosensor based on Au-mesoporous silica nanoparticles (Au-MSN) after selecting a 37-mer aptamer sequence against the opiate alkaloid codeine. Here, thiolated codeine aptamers were conjugated to Au-MSN's immobilized on the surface of a glassy carbon electrode. Changes in electrical impedance at the electrode surface associated with aptamer-target binding were used to monitor codeine concentrations across a linear range of 10 pM to 100 nM. Furthermore, denaturation of the aptamer sequences by incubation with heated distilled water allowed the sensor to be reused for subsequent measurements.

With relatively fast selection and isolation times compared to antibodies (i.e. a matter of weeks), nucleic acid aptamers also represent a great opportunity to rapidly develop forensic assays for the detection of new psychoactive substances (NPS). These substances (typically scheduled drugs slightly modified to circumvent legal restrictions) are emerging at an unprecedented rate, with over 450 new compounds identified by the EU early warning system since 2005 [109]. At present, the majority of NPS compounds are poorly or not detected by standard immunoassay tests, forcing analysts to rely on mass-spectrometry techniques [110]. Despite the excellent potential of aptasensor platforms as drug-screening assays, aptamers selected towards any NPS target have yet to be reported in the literature.

4.3 Forensic toxicology

The synthetic nature of SELEX protocols means that aptamer recognition moieties may be selected against toxic compounds that would likely kill animal hosts during standard *in vivo* antibody-generation methods [111]. Such moieties may be therefore of great benefit to toxicology practices, where determining cause of death is often dependent upon the detection and quantitation of hazardous substances. This branch of forensic chemistry heavily relies on the use of expensive and labor-intensive analytical techniques to confirm the identify of various poisons and toxins [112].

Fig. 5 – Schema of a graphene-quenching fluorescence aptasensor for the detection of ochratoxin A developed by Sheng *et al.* [113]. Here, aptamers are made initially fluorescent via 5' labeling with 6-carboxyfluorescein (6-FAM). Under normal conditions, these aptamers are readily adsorbed onto the basal plane of PVP-protected graphene oxide, which subsequently quenches 6-FAM emission by energy transfer. However, in the presence of ochratoxin A, antiparallel G-quadruplex formation prevents such adsorption and fluorescence signals are generated. Using this sensor, Sheng *et al.* were able to monitor ochratoxin A with high selectivity in a liner range from 50-500 nM.

Ochratoxin A (OTA) is a toxic metabolite secreted by *aspergillus* and *penicillium* fungi species that can exert severe nephrotoxic, immunotoxic, and carcinogenic effects [114]. As such toxins represent a threat to health through the contamination of commercialized food systems, significant efforts have been made towards the development of a simple and flexible sensing platform for the detection of OTA compounds [115]. In a strategy designed by Sheng *et al.* (Fig. 5), this sensing was achieved by the use of FAM-modified aptamers which, in the absence of OTA, are adsorbed onto a basal plane of graphene oxide and quenched [113]. However, in the presence of target molecules, aptamers are induced into particular three-dimensional confirmations and resist adsorption, allowing fluorescence to be monitored.
In an extension of the Au-NP aggregation assay exploited by Yarbakht and Nikkhah for the detection of amphetamines, Wu *et al.* also utilised an aptamer-based sensor for the detection of arsenic within aqueous solutions [116]. In this method, a cationic surfactant Hexadecyltrimethylammonium bromide (CTAB) was employed instead of salts to stimulate nanoparticle aggregation, allowing the colorimetric detection of arsenic concentrations in the range of 1-500 parts per billion.

Work from Lamont *et al.* resulted in the selection of an aptamer (SSRA1) specific to the B-chain of ricin [117]. Ricin, a cytotoxic protein of biological origin, is considered as a potential threat agent for terrorist use because of its high toxicity and relative availability. The selected aptamer displayed superior detection capabilities compared to a commercially available ELISA kit for ricin. Following this study, attempts have been made in order to embed the SSRA1 aptamer into a biosensing platform for rapid and sensitive ricin detection. Esteban-Fernández de Ávila *et al.* reported on a micromotor sensing strategy for the fluorescent detection of ricin [118]. Self-propelled reduced graphene-oxide (rGO)/platinum (Pt) micromotors were synthesized and were modified with the ricin-specific aptamer tagged with a fluorescent dye. The resulting complex is non-fluorescent due to the quenching effect of the graphene surface. In the presence of the target toxin, the aptamer is displaced from the graphene-oxide quenching motor surface and its fluorescence is restored. By this method, real-time detection of trace amounts of ricin toxin could be achieved in biological and environmental samples. Li *et al.* further developed a detection system for ricin based on isothermal strand-displacement polymerase reaction [119]. The ricin-specific aptamer SSRA1 was first hybridized with a single stranded DNA blocker and then it was immobilized on the surface magnetic beads. When ricin binds to the aptamer, the blocker is released and it hybridizes to with a dye-modified hairpin probe triggering an isothermal strand-displacement polymerase reaction. The fluorescent double stranded DNA product cannot be quenched as it does not interact with the graphene oxide surface, resulting in an increase of the fluorescence intensity.

Scientific efforts have been made in the forensic field towards the development of new methods for the detection of chemical weapon agents with emphasis been placed on the real-time, on-site analysis of nerve agents (e.g. sarin). Zhao *et al.* developed a cantilever-based aptasensor for dimethyl methylphosphonate (DMMP), a nerve agent simulant [120]. The piezoresistive cantilever sensor was designed with four cantilevers (two sensing and two reference cantilevers). A biotinylated anti-DMMP aptamer was immobilized on the surface of the sensing cantilevers. In the presence of DMMP, binding takes place between the immobilised aptamers and the target; this results in a surface stress and a bending of the sensing cantilevers which can be recorded and measured. The method resulted in sensitive (50 nM–1.0 μM) and specific detection of DMMP in aqueous solutions.

4.4 Explosives

The development of rapid, cost-effective and reliable assays for the detection of explosive molecules in both aqueous and gaseous samples is a high priority for forensic investigators, counter-terrorism agencies and global de-mining projects [121]. Ehrentreich-Förster *et al.* argue that aptamers are likely to make ideal recognition moieties for such assays, as immunological methods are often disadvantaged by the poor specificity of antibodies raised against low molecular weight explosive analytes [122]. Furthermore, aptamers are known to retain their activity in a number of organic solvents (such as methanol and acetonitrile) commonly used for the solubilisation and detection of explosive compounds.
To prove this concept the same group demonstrated the construction of an optical aptasensor towards the detection of 2,4,6-trinitrotoluene (TNT). In this sensing strategy, activated TNT derivatives are first immobilized on the surface of silanised optical fibres. TNT-specific aptamers mixed within sample solutions are then introduced into the flow cell and bind to the surface-bound analytes unless already captured by TNT present within the sample itself (Fig. 6). Nanobeads covalently attached to these aptamer sequences are then used to report the amount of ligands bound to the fibre (with emission intensities inversely proportional to the concentration of TNT present in measured samples).

Fig. 6 – Schema of an optical fibre-based aptasensor for the detection of TNT developed by Ehrentreich-Förster *et al.* [122]. Silanised optical fibres functionalized with TNT competitors are first embedded within the surface of a

flow cell. Under normal conditions, a sample solution containing fluorescently labeled aptamers is introduced into the cell, which subsequently bind to the competitive analogues. Excitation by a laser at 480 nm allows the detection of aptamer presence on the fibre. However, a reduction in signal occurs when free TNT molecules present within the sample solution capture available aptamers, preventing interaction with the surface-attached analogues.

Whilst aptasensing assays towards explosive targets other than TNT remain extremely limited, it is hoped that this review will encourage researchers in the field explosives research to consider their use for the detection for compounds such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX).

5. Conclusions

Challenges associated with the immunological and instrumental analysis methods currently employed within both crime scene and laboratory-based testing procedures have resulted in the demand for new techniques able to allow the rapid, sensitive and specific detection of forensically relevant analytes. In this review, we have highlighted the potential of biosensing technology that incorporates aptamer recognition moieties to address this demand. Moreover, we have reported on a wide range of aptasensors that, although being successfully developed for use in other analytical disciplines, could be readily adapted to forensic analysis.

Whilst research in the development of aptasesnsors for forensic purposes is extremely limited, it is hoped that recent technological advances (e.g. NGS) will make aptamer-based sensor development more accessible to researchers working within the field of forensic science.

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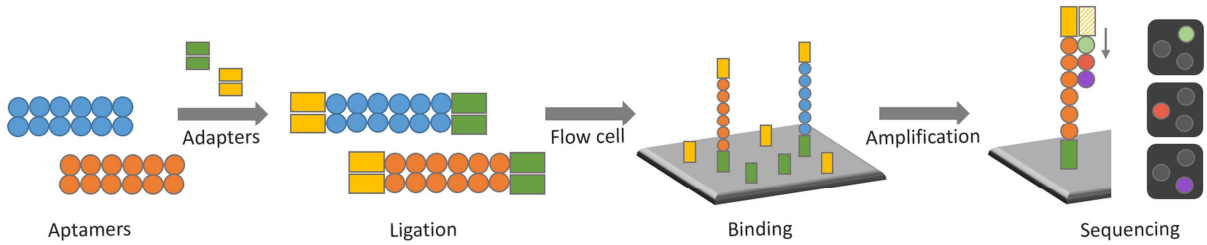

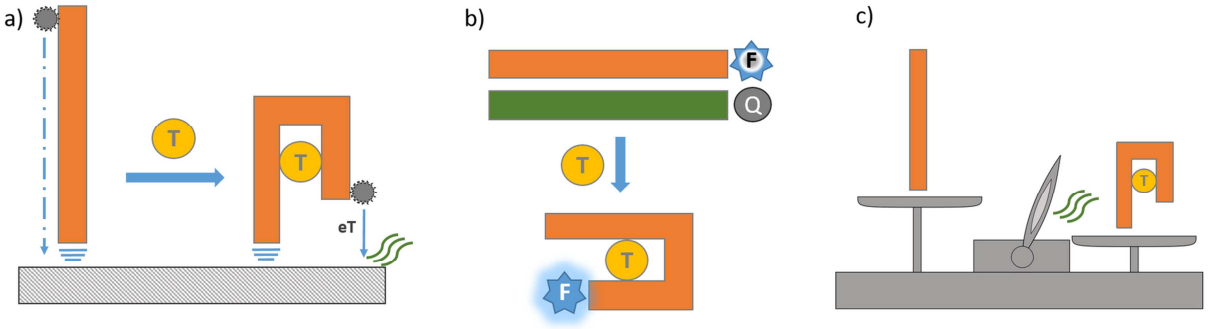

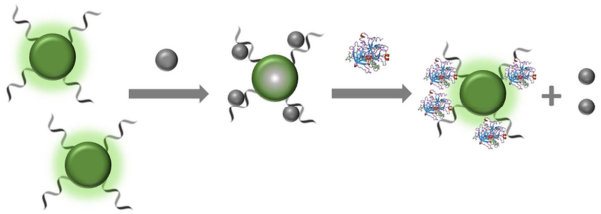

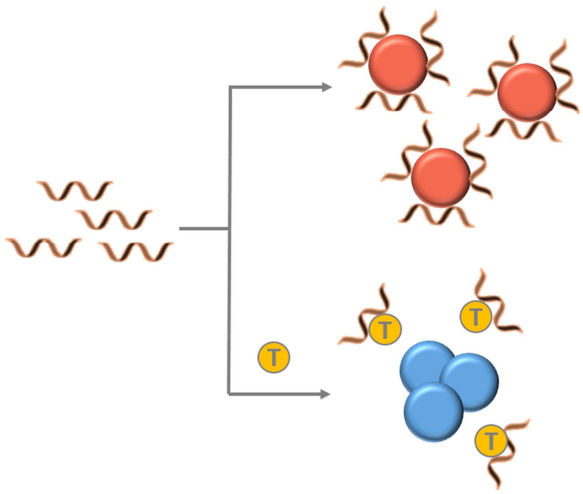

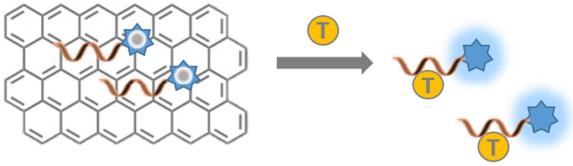

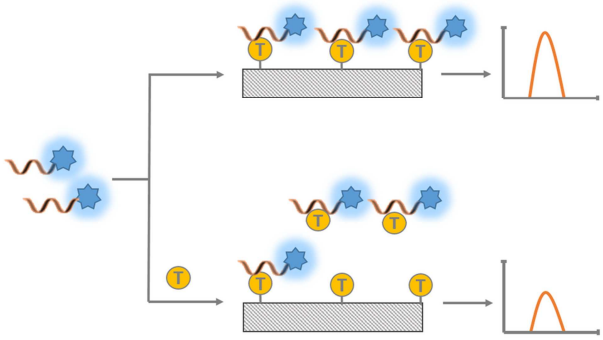

Highlights

- The current state of aptasensor development processes is outlined
- Recent advances in aptamer selection, sequencing techniques and affinity testing are reported
- Sensing mechanisms applicable to forensic analysis are critically evaluated
- Forensic disciplines likely to benefit from aptasensor development are identified